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Drug or tool, design or serendipity?

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Iterative protein structure-based ligand design has led to a 'selective' inhibitor of human non-pancreatic secretory phospholipase A_2 which provides a new tool for probing metabolic pathways and may lead to a useful drug.

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Blocking human non-pancreatic secretory phospholipase A_2 (hnps-PLA_2) may open new doors to the 4.3 billion dollars world market[1] of non-steroidal anti-inflammatory drugs (NSAIDs). At least this has been the hope of many pharmaceutical companies for more than twenty years. Thus far, their scramble for this potentially massive financial reward has been in vain[2]. However, in this issue of *Nature Structural Biology* Schevitz *et al.*[3], researchers at Eli Lilly, report that they have crafted a potent inhibitor of hnps-PLA_2 that displays 1500-fold selectivity when assayed against pancreatic PLA_2. They have based their strategy on a clever screening assay and a process of iterative protein structure-based ligand design. An important lesson of their successful design is that it is rooted in a 'common sense' approach rather than elaborate computational methods. It remains to be seen, however, whether the new inhibitor will ever make it onto the market as a drug. That will depend on pertinent pharmacodynamic properties. Perhaps the biggest significance of the inhibitor is that it will be a new tool for unraveling the pharmacological importance of the biochemical pathways on which PLA_2 operates. The big question remains, though: can the successful rational approach followed by the Eli Lilly researchers be applied to other proteins as well, or does the process of selective design still involve some considerable amount of luck?

PLA_2 pharmacology

Phospholipase A_2 enzymes hydrolyze the *sn*-2 ester bond of membrane phospholipids, liberating free fatty acids and lysophospholipids. Pharmacological interest in this reaction stems from the belief that PLA_2-catalyzed release of arachidonic acid is the rate-limiting step in the biosynthesis of potent mediators of inflammation and allergy, such as prostaglandins, thromboxanes and leukotrienes[2] (Fig. 1). Control of PLA_2 activity should translate into diverse medical applications, such as relief for patients with rheumatoid arthritis, and increased survival chances in cases of endotoxic shock or pancreatitis[4].

Unfortunately, the existence of multiple forms of PLA_2 complicates the understanding of the cellular mechanisms of arachidonic acid production. In humans there are two 14,000 M_r secretory PLA_2s, one produced in the pancreas and the other found in inflammatory cells and exudates, this latter enzyme being referred to as hnps–PLA_2. Both enzymes are not specific for arachidonic acid-containing phospholipids, and their activity is not regulated by allosteric effectors. In contrast, there is a 87,000 M_r cytosolic PLA_2 with a clear preference for arachidonic acid phospholipids[5], of which the activity is regulated by phosphorylation[2]. Because there are no inhibitors known that selectively block only one of the PLA_2s the implication of hnps–PLA_2 in inflammation is based on circumstantial evidence rather than facts[6]. The selective inhibitor designed by Schevitz *et al.* may resolve this issue.

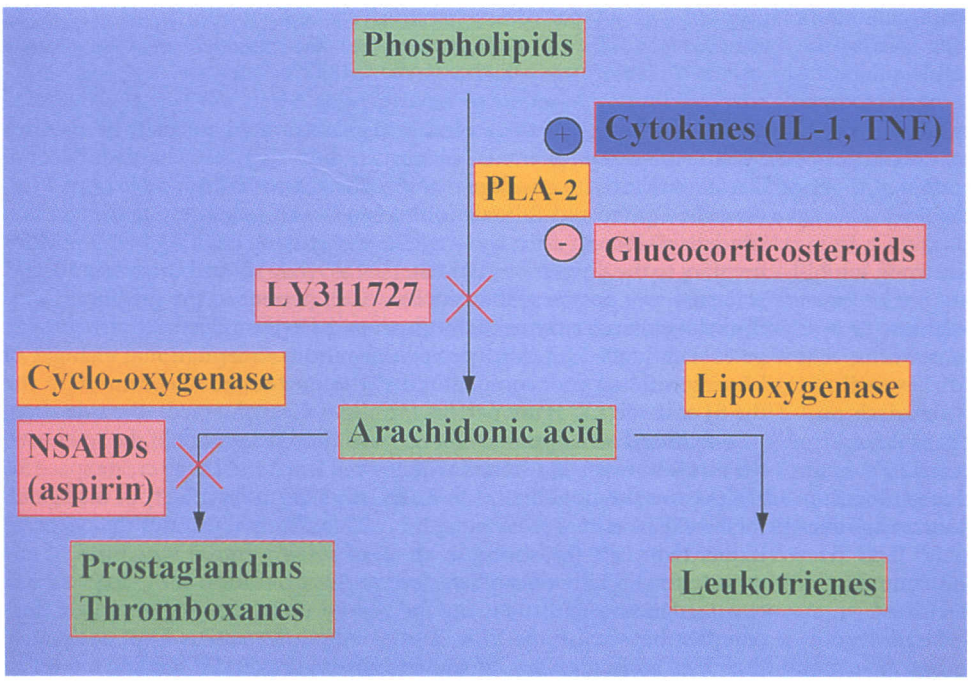

Fig. 1 Traditional view of the inflammation cascade. Cytokines, such as interleukin–1 or tumour necrosis factor, induce synthesis and secretion of PLA_2. This synthesis can be prevented by the administration of glucocorticosteroids. PLA_2 hydrolyzes membrane phospholipids liberating arachidonic acid. This step can be blocked by the new inhibitor LY311727, designed by Schevitz *et al.*[3]. Arachidonic acid can be metabolized by cyclo–oxygenases, a step that can be inhibited by aspirin, or by 5–lipoxygenases. This metabolism gives rise to potent mediators of inflammation, such as prostaglandins, thromboxanes and leukotrienes.

Lead discovery

The first step in the precarious undertaking of drug design is to find a good lead molecule. In principle, a lead is a molecule with micromolar affinity, or activity, that lends itself to easy chemical modification. Current approaches to find leads are based on screening of many compounds, usually of the order of 100,000. If the three-dimensional structure of the target protein is known computer-based methods can reduce the screening efforts to about 100 compounds in favourable cases[7].

The researchers at Eli Lilly eschewed computers and went for the traditional screening method. In two and a half years they screened about 90,000 compounds against hnps-PLA_2. Many of the compounds were evaluated in three different assays: a 'quick and dirty' *Eschericha coli* assay, a robust chromogenic assay, and a response test in lung tissue. Great care was taken to evaluate PLA_2 inhibition in an appropriate manner. Because PLA_2 operates at a lipid/water interface this is far from trivial, but the relevant protocols have been established[8]. The tissue assay was crucial to verify that the inhibitors would not be active *in vivo* for the wrong reasons, namely by inhibiting cyclo-oxygenase, an enzyme downstream in the inflammation cascade (Fig. 1). To their surprise, Schevitz *et al.* found a molecule that is very similar to indomethacin, a known NSAID.

Indomethacin is a strong inhibitor of cyclo-oxygenase, but had been shown to weakly block hnps-PLA_2 as early as 1978[9]. It is noteworthy that indomethacin was discovered in a serotonin antagonist research program by researchers at Merck in 1963[10]. This program had been started in 1957 because of the incorrect belief that serotonin was a mediator of inflammation[11]. This illustrates that it is not uncommon for drugs to be discovered for the wrong reasons, and that good leads for current drug–design programs may have been around for decades.

Lead optimization

During lead optimization several desirable properties of a lead are improved. The first goal is to increase the affinity of the molecule because this should guarantee a high degree of selectivity, an adage that is described in an old folktale: only Cinderella's foot is perfectly complementary to the slipper she lost at the ball. Just as a person will avoid an ill-fitting shoe, an enzyme that competes with hnps–PLA_2 for inhibitor binding will be a poor competitor if the fit of the ligand is not perfect.

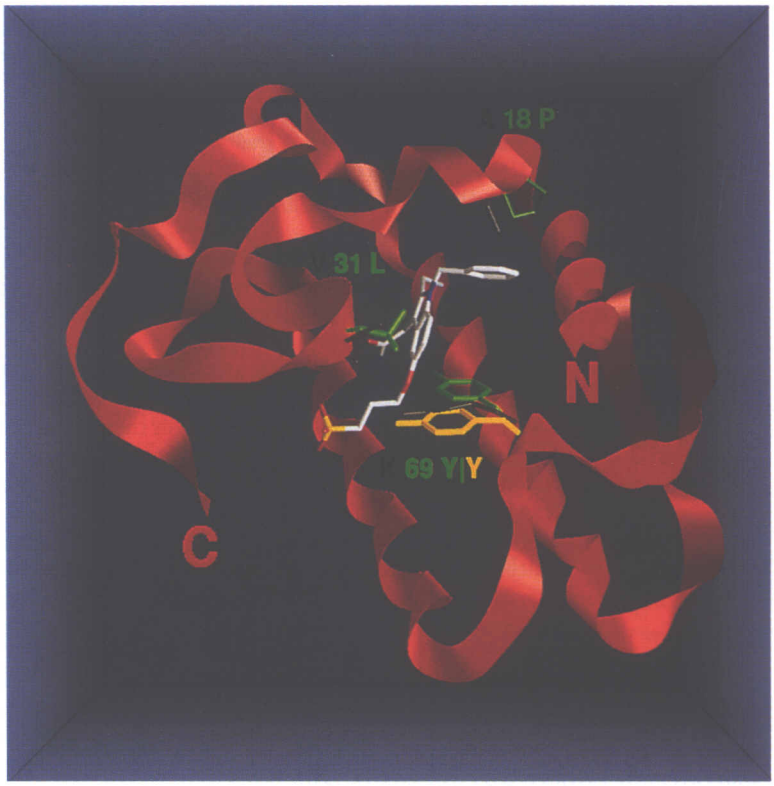

Fig. 2 Hnps–PLA_2 and porcine pancreatic PLA_2. Ribbon diagram of hnsp–PLA_2 (red; N and C termini are indicated) with side chains of residues that may explain the discrimination between hnsp–PLA_2 (black side chains) and porcine pancreatic secretory PLA_2 (green side chains) by LY311727 (molecule in the centre of the figure). The Tyr 69 side chain of the porcine enzyme has to move out of the way in order to accommodate the inhibitor. This postulated conformation (yellow) has in fact been observed for a mutant of the porcine enzyme in the presence of an amide substrate analogue[16]. Figure made with the program GRASP[23].

Traditional lead optimization relies on synthesis of large series of derivatives in the hope that a few of them will have increased potency. Rather than take this shot-gun approach, the Eli Lilly researchers determined the crystal structure of the complex between hnps-PLA_2 and their lead molecule. This led to two surprises. First, the carboxylic-acid function of the lead makes a direct hydrogen bond to the carboxylate moiety of Asp 49. Docking methods would never have found this binding mode because both chemical groups would normally be assumed to be negatively charged. Second, there is no Ca^{2+} bound to hnps-PLA_2 in this complex despite the fact that the enzyme has a K_m of 1.5 mM for Ca^{2+} (ref. 12) and there was 10 mM Ca^{2+} present in the medium. Compared to the unliganded structure[13,14] several conformational changes of the protein to accommodate the ligand are observed: the side chain of His 6 swings out of the active site, and Leu 2 and Lys 69 create space for the 5-methoxy moiety of the ligand. This clearly illustrates the necessity of solving crystal structures of enzyme-inhibitor complexes as our poor understanding of protein flexibility prevents us from adequately predicting ligand binding modes.

In the process of lead optimization Schevitz *et al.* again made no use of modern computational methods[15] where the addition of thousands of substituents to a lead is evaluated for complementarity to the

Table 1 Residues in contact[1] with LY311727

Residue No.	hnps–PLA_2	pps–PLA_2 [2]	hps–PLA_2 [3]	Note
2	Leu	Leu	Val	
5	Phe	* [4]	*	
6	His	Arg	Arg	swings out for inhibitor binding
9	Ile	*	*	
18	Ala	Pro	Pro	
22	Tyr	Phe	Tyr	backbone and CB atoms in contact
23	Gly	Asn	Asn	
28	His	Tyr	Tyr	backbone atoms in contact
29	Cys	*	*	
30	Gly	*	*	
45	Cys	*	*	
48	His	*	*	
49	Asp	*	*	
52	Tyr	*	*	
69	Lys	Tyr	Tyr	
106	Phe	*	*	

[1]Contact is defined as the sum of the van der Waals radii plus a tolerance of 1.0 Å;[2] pps = porcine pancreatic secretory; [3] hps = human pancreatic secretory; [4]* means that the residue is identical to the one in hnps–PLA_2.

target enzyme. As before, they went for a 'common sense' approach by mimicking what was already known about PLA_2 biochemistry. The Eli Lilly researchers successfully pasted onto their lead the two Ca^{2+} ligand moieties of the potent non-selective PLA_2 inhibitor (*R*)–2–dodecanoyl–amino–1–hexanol–phosphoglycol, for which the binding mode is known[16]. Again, determining the crystal structure of the complex with the new ligand proved to be extremely informative. For example, the 5-methoxy group of the new inhbitor moved by 5 Å compared to its position in the complex of PLA_2 with the original lead molecule. Such major shifts of lead molecules have now been seen several times by protein crystallographers: similar ligands do not necessarily bind in similar modes[17,18].

After four rounds of structure-based ligand design, with a turn-around time per cycle of about three weeks, Schevitz *et al.* obtained a ligand, LY311727, which was 750 times more potent than their lead against hnps-PLA_2.

Selectivity

The Eli Lilly researchers announce that their inhibitor is 1,500 times more potent against hnps–PLA_2 than against porcine pancreatic PLA_2. However, nowhere in their strategy did they take the structure of the pancreatic enzyme into account. The obtained selectivity seems to be only a consequence of the aim for maximal potency and, therefore, maximal complementarity to hnps–PLA_2. Can we understand the origin of this selectivity?

We docked the selective Eli Lilly inhibitor LY311727 in the structure of hnps–PLA_2, as observed in the presence of a transition state analogue[14], by means of the program BIOGRAF[19]. Next, we verified the docking mode by careful visual comparison with the stereo figure in the paper by Schevitz *et al* (the modelling step was necessary since the coordinates of the Eli Lilly PLA_2 complexes are on hold). Subsequently, the structure of a mutant porcine pancreatic PLA_2 as observed in the presence of an amide substrate analogue[16] and wild–type porcine PLA_2 in the absence of a ligand[20] were superimposed on the hnps–PLA_2 structure (Fig. 2). Comparison of residues whose side chains contact the inhibitor does not give a clear–cut reason why the inhibitor should be selective. The Ala 18 residue contacts the benzyl system of LY311727. The equivalent Pro residue of the porcine enzyme is about 1 Å further away, which may lead to poor packing of the benzyl substituent in the molecular complex. Val 31 of hnsp–PLA_2 makes hydrophobic interactions with the ring system of the inhibitor. The equivalent Leu of porcine PLA_2 would bump into this ring system and should therefore rotate away to avoid the clash. This would amount to exposing part of the Leu side chain and part of the ring system of LY311727 to solvent, for which an entropic energy penalty would have to be paid. Tyr 69 of porcine PLA_2 provides the same contacts as Lys 69 of hnps–PLA_2 in the complex structures; at least, in the 'swung out' conformation as observed in the presence of the amide substrate analogue[16]. In the absence of a ligand the Tyr side chain is 'swung in'[20] and would preclude binding. Since such a conformational change is vital for the functioning of the enzyme it seems likely that the associated energetic cost is minimal. In conclusion, one has to assume that the selectivity factor of 1,500 would have to be ascribed to the differences at residues 18 and 31.

A second question that arises is whether selectivity against the porcine pancreatic enzyme is relevant, because a drug will have to deal with the human pancreatic enzyme. Direct testing against this enzyme is a problem since the enzyme is not readily available. A sequence alignment (Table 1) demonstrates that of the 16 residues that presumably contact the inhibitor one is different between the porcine and human pancreatic enzyme: porcine Leu 2 is a Val in the human PLA2. Leu 2 makes hydrophobic interactions with both the benzyl system and the indole ring of the inhibitor. Obviously the smaller Val side chain will make less extensive interactions. Therefore, LY311727 should be even more selective when tested against human pancreatic PLA2.

The road to drug

How far away are the researchers at Eli Lilly from an effective drug? According to Connolly and Robinson[21] a hnps–PLA2 inhibitor should:

- possess PLA2 specificity *in vitro* and the IC50 should be better than 1 μM for enzyme and cellular assays;
- exhibit *in vivo* anti–inflammatory activity in animals sensitive to NSAIDs, 5–lipoxygenase inhibitors or platelet activiating factor (PAF) antagonists;
- be orally active with a therapeutically effective dose in 50 % of the subjects (ED50) smaller than 10 mg kg^{-1};

•reduce multiple lipid mediators in inflammatory exudates or tissues.

Only the first condition has been demonstrated for LY311727 at present, while there are indications that the last condition is met from lung strips assay[3]. The second and third factors are strongly related to the pharmacodynamics issues of absorption, distribution in the body, metabolism and excretion. For example, most of the peptidomimetics developed by protein structure-based design as HIV protease inhibitors have extremely short serum half-lives, less than an hour, and are therefore of no therapeutic use[22]. Even if there are no problems with the pharmacodynamic properties of a molecule one still does not have a drug. Many promising molecules do not survive the stringent criteria of extensive toxicological testing.

Genesis of a tool

From the work by Schevitz *et al.* it is clear how valuable a cyclical process of structure-based design is for lead optimization. It also highlights the current problems of purely computational design: a poor understanding of the energetics of molecular interactions, difficulties to handle conformational flexibility, and a lack of predictive tools for pharmacodynamics and toxicology[15]. Without any doubt LY311727 is an extremely useful tool to study the role of secretory phospholipases in the inflammation process. It's future as a route to an effective drug is less clear.

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